



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 368 486

A2

(19)

(12)

(21) Application number: 89310620.3

(22) Date of filing: 17.10.89

(51) Int. Cl.5: C07K 5/08, C07K 7/06,
C07K 7/10, C12P 21/00,
C12N 15/00, A61K 37/02,
C07K 13/00

(30) Priority: 10.11.88 US 269583

(43) Date of publication of application:
16.05.90 Bulletin 90/20

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: MERCK & CO. INC.
126, East Lincoln Avenue P.O. Box 2000
Rahway New Jersey 07065-0900(US)

Applicant: THE TRUSTEES OF THE
UNIVERSITY OF PENNSYLVANIA
c/o Office of Research Administration 133
South 36th Street Suite 300
Philadelphia Pennsylvania 19104-3246(US)

(72) Inventor: Shattil, Sanford J.
510 Merwyn Road
Narberth Pennsylvania 19072(US)
Inventor: Taub, Rebecca A.
403 Great Spring Road
Rosemont Pennsylvania 19010(US)
Inventor: Friedman, Paul A.
403 Great Spring Road
Rosemont Pennsylvania 19010(US)

(74) Representative: Stebbing, Peter John Hunter
Stebbing & Co. 45 Lancaster Mews
Lancaster Gate, London W2 3QQ(GB)

(54) Novel inhibitors of platelet binding.

EP 0 368 486 A2 (57) Peptides comprising the tripeptide sequence of arginine-tyrosine-aspartic acid, linked from amino- to carboxy-terminus, are inhibitors of fibrinogen binding, platelet aggregation, glycoprotein IIb-IIIa binding and related aberrant and normal physiological conditions.

Novel Inhibitors of Platelet Binding

BACKGROUND OF THE INVENTION

Many adhesive proteins, including fibrinogen, fibronectin, von Willebrand factor, and vitronectin, contain an amino acid sequence that functions as a recognition site for adhesion receptors. The amino acid sequences within these proteins are recognized by structurally-related receptors on a variety of mammalian cells that have been termed integrins or cytoadhesions. Hynes, Cell, 48, 549-554 (1987); Ruoslahti et al., Science, 238, 491-497 (1987).

One well-characterized member of the integrin family is the blood platelet membrane glycoprotein IIb-IIIa complex (GP IIb-IIIa). Phillips et al., Blood, 71, 831-843 (1988). Upon platelet activation, GP IIb-IIIa becomes competent to bind fibrinogen, a process required for platelet aggregation. Bennett et al., J. Clin. Invest., 64, 1393-1400 (1979); Marguerie et al., J. Biol. Chem., 254, 5357-5363 (1979). Based on studies with synthetic peptides, the Arg-Gly-Asp-Ser sequence at positions 572-575 of the fibrinogen A α chain appears to play a major role in the interaction of this protein with GP IIb-IIIa, although other regions in fibrinogen may be involved as well. Gartner et al., J. Biol. Chem., 260, 11891-11894 (1985); Ginsberg et al., J. Biol. Chem., 260, 3931-3936 (1985); Haverstick et al., Blood, 66, 946-952 (1985); Pytela et al., Science, 231, 1159-1162 (1986); Kloczewiak et al., Thrombosis Res., 29, 249-255 (1983); Kloczewiak et al., Biochemistry, 23, 1767-1774 (1984). However, the precise details of the molecular interaction between fibrinogen and GP IIb-IIIa are, at present, unknown. The tripeptide sequence Arg-Gly-Asp has also been identified in the adhesive proteins fibronectin, von Willebrand factor and vitronectin. Hynes, Cell, 48, 549-554 (1987); Ruoslahti et al., Science, 238, 491-497 (1987). A similar region of fibronectin containing the sequence Arg-Glu-Asp-Val has been implicated in the binding of this protein to melanoma cells. Humphries et al., J. Cell Biol., 103, 2637-2647 (1986). Moreover, von Willebrand factor, which can bind to GP IIb-IIIa on activated platelets, contains, in its long (significantly greater than 50 residue) amino acid sequence, an Arg-Tyr-Asp-Ala, in addition to an Arg-Gly-Asp-Ser, sequence. Titani et al., Biochemistry, 25, 3171-3184 (1986). Furthermore, the sequence Arg-Phe-Asp-Ser and Arg-Tyr-Asp-Ser are found within the long chain peptides that comprise the major histocompatibility antigens, peptides which have a length greatly in excess of 50 amino acid residues. Auffray et al., Human Immunol., 15, 381-390 (1986).

Numerous applications have been found for peptides containing the amino acid sequence Arg-Gly-Asp. The binding of fibrinogen to blood platelets, for example, has been found to be inhibited by

- 5 Arg-Gly-Asp-containing peptides. In addition, PAC1, an IgM-x murine monoclonal antibody that, like fibrinogen, appears to bind to GP IIb-IIIa on activated platelets, is similarly inhibited by such peptides. The inhibition of fibrinogen and PAC1 occurs at an apparent Ki of about 10-20 μ M when the tetrapeptide Arg-Gly-Asp-Ser is employed. Shattil et al., J. Biol. Chem., 260, 11107-11114 (1985); Bennett et al., J. Biol. Chem., (1988); Shattil et al., Blood, 68, 1224-1231 (1986). Moreover, U.S. Patent No. 4,683,291 discloses the usefulness of peptides containing the Arg-Gly-Asp sequence in inhibiting cellular adhesion in general, and platelet to platelet binding specifically. Such peptides are also reported as useful in retarding the formation of blood clots.

Additional and/or better peptides which meet the important ends of inhibiting fibrinogen to platelet binding, platelet to platelet binding and/or blood clot formation are needed. The present invention is directed to these and other important needs.

SUMMARY OF THE INVENTION

- 30 The present invention provides compounds of the general Formula I

$$R^1-(X^1)_n\text{-Arg-Tyr-Asp-(X}^2\text{)}_m\text{-R}^2 \quad (\text{I})$$
 and physiologically acceptable salts thereof, wherein:
 X¹ and X², independently, are 1 to about 50 amino acid residues, provided that the total number of amino acid residues in X¹ and X², taken together, is less than or equal to about 50;
- 35 m and n, independently, are 0 or 1;
 R¹ is a protected or unprotected terminal amino group; and
 R² is a protected or unprotected terminal carboxy group.
- 40 Also included in the subject invention are compounds of the general Formula II

$$R^1-(X^1)_n\text{-Arg-Tyr-Asp-(Y)}_o\text{-}(X}^2\text{)}_m\text{-R}^2 \quad (\text{II})$$
 and physiologically acceptable salts thereof, wherein:
- 45 X¹ and X², independently, are 1 or more amino acid residues;
 Y is 1 or more amino acid residues, provided that Y does not include a Ser or Ala residue;
 m, n and o, independently, are 0 or 1, provided that when m is 1, o must be 1;

R¹ is a protected or unprotected terminal amino group; and
R² is a protected or unprotected terminal carboxy group; and wherein said compound inhibits fibrinogen binding, platelet aggregation, glycoprotein IIb-IIIa binding, thrombosis and/or cancer metastasis.

The invention further provides compositions comprising one or more of the foregoing compounds, and methods of using such compounds or compositions in inhibiting fibrinogen binding, platelet aggregation, and/or glycoprotein IIb-IIIa binding. The subject compounds or compositions are also effective in the treatment of certain physiological conditions, such as thrombosis and/or cancer metastasis, and the present invention is further directed to these uses.

Also included within the ambit of the present invention are monoclonal and polyclonal antibodies directed to the subject compounds and nucleic acid fragments comprising nucleotide sequences which encode the subject compounds.

Finally, the present invention comprehends a peptide comprising an amino acid sequence corresponding to the amino acid sequence of the variable region of the μ heavy chain of monoclonal antibody PAC1, as well as antibodies incorporating that sequence. The present invention further includes a peptide comprising an amino acid sequence corresponding to the amino acid sequence of the variable region of the μ heavy chain of monoclonal antibody PAC1, and an amino acid sequence corresponding to the amino acid sequence of the variable region of the μ light chain of monoclonal antibody PAC1, as well as antibodies incorporating that sequence. Most specifically, the invention is directed to the monoclonal antibody PAC1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of the variable region of the χ light chain of a PAC1 hybridoma cell, with nucleotide numbering shown above. The site of the V to J region junction is indicated. The predicted amino acid sequence, along with the amino acid numbers appear on the next two lines below. The amino acids are numbered according to convention. Kabat E.A., Wu, T.T., Reid-Miller, M., Petty, H.M., Gottesman, K.S., Sequences of Proteins of Immunologic Interest (US DHHS, PHS, NIH 1987). The complimentary determining regions (CDRs) are shown in boxes.

Figure 2 shows the nucleotide sequence of the variable region of the μ heavy chain of a PAC1 hybridoma cell with nucleotide numbering shown

above. The site of the V to J region junction is indicated. The predicted amino acid sequence, along with the amino acid numbers appear on the next two lines below. The amino acids are numbered according to convention. Kabat E.A., Wu, T.T., Reid-Miller, M., Petty, H.M., Gottesman, K.S., Sequences of Proteins of Immunologic Interest (US DHHS, PHS, NIH 1987). The complimentary determining regions (CDRs) are shown in boxes. CDR3 is comprised of the amino acid sequence Arg-Ser-Pro-Ser-Tyr-Tyr-Arg-Tyr-Asp-Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr.

Figure 3 shows the effect of a 21 amino acid peptide derived from the variable region of the PAC1 μ heavy chain (hereinafter referred to as "the Arg-Tyr-Asp-21mer peptide") on the binding of PAC1 and fibrinogen to activated human blood platelets. The Arg-Tyr-Asp-21mer has the sequence Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr-Arg-Tyr-Asp-Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr. Gel-filtered platelets were incubated for 15 min with ADP (10 μ M), and epinephrine (10 μ M), in the presence of various peptides and either FITC-PAC1 (Figure 3A) or FITC-9F9 (Figure 3B) and fibrinogen (50 μ g/ml). The specific peptides tested were Arg-Tyr-Asp-21mer (filled circles), Arg-Gly-Asp-Ser (unfilled circles) and an unrelated-28mer (control; unfilled squares). Binding was determined by flow cytometry. Antibody binding is expressed as the percent of binding observed in the absence of added peptide. Error bars represent the mean \pm SEM of 5 separate experiments. In Figure 2A, the data points without error bars represent the mean of two experiments.

Figure 4 shows the effect of the Arg-Tyr-Asp-21mer peptide on the binding of various monoclonal antibodies to platelets. Gel-filtered platelets were incubated in the presence of Arg-Tyr-Asp-21mer and various FITC-labelled anti-platelet monoclonal antibodies. Monoclonal antibody binding was determined by flow cytometry. In the case of antibody AP1 (specific for platelet glycoprotein Ib) (unfilled circles), the platelets were not activated. In the case of antibodies PAC1 (specific for activated glycoproteins IIb-IIIa) (filled circles), B1B5 (specific for glycoprotein IIb) (filled squares) and S12 (specific for GMP-140) (unfilled triangles), the platelets were activated with ADP and epinephrine (10 μ M each). The data shown are from a single experiment representative of two runs performed as described.

Figure 5 shows the effect of Arg-Tyr-Asp-21mer on platelet aggregation. Gel-filtered platelets were stirred for 3 min in an aggregometer cuvette in the presence of ADP (10 μ M), fibrinogen (100 μ g/ml), CaCl₂ (1mM), and one of the following: Arg-Tyr-Asp-21mer (filled circles); Arg-Tyr-Asp-21mer having a Gly substituted for the Tyr residue

(Arg-Gly-Asp-21mer) (filled squares); Arg-Tyr-Asp-21mer having the Asp and Arg residues reversed (Asp-Tyr-Arg-21mer) (filled triangles); or Arg-Tyr-Asp-21mer having a d-Ala residue substituted for the Tyr residue (Arg-d-Ala-Asp-21mer) (unfilled circles). Aggregation is expressed as the initial rate of aggregation, where the rate of aggregation in the absence of added peptide is arbitrarily denoted as 100%. The data represent the means of three separate experiments performed as described.

Figure 6 shows the effect of Arg-Tyr-Asp on the binding of FITC-PAC1 (Figure 6A) or FITC-9F9 (Figure 6B) and fibrinogen to platelets stimulated with ADP and epinephrine (10 μ M each). The specific peptides tested were Arg-Tyr-Asp-21mer (filled circles), Arg-Gly-Asp-21mer (filled squares), Asp-Tyr-Arg-21mer (filled triangles), Arg-d-Ala-Asp-21mer (unfilled circles) or Arg-Gly-Asp-Ser (unfilled squares). The conditions in each of the binding assays were identical to those in Figure 3. Data shown are from a single experiment representative of three runs performed as described.

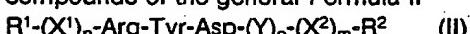
DETAILED DESCRIPTION OF THE INVENTION

The present invention involves the discovery of novel and very active compounds that contain the tripeptide sequence of arginine-tyrosine-aspartic acid, linked from amino- to carboxy-terminus. The subject invention derives from the surprising finding that the arginine-tyrosine-aspartic acid sequence is present in one of the heavy chain hyper-variable regions of PAC1 murine monoclonal immunoglobulin, and in that position is a potential mediator in the binding of this monoclonal antibody to the blood platelet membrane fibrinogen receptor, glycoprotein IIb-IIIa.

More specifically, the present invention encompasses peptide compounds of the general Formula I



and physiologically acceptable salts thereof. The present invention further encompasses peptide compounds of the general Formula II



and physiologically acceptable salts thereof. These compounds are characterized by their inhibitory potency, namely their ability to retard or prevent the following: the binding of the adhesive protein fibrinogen to blood platelets (referred to herein as inhibiting "fibrinogen binding"); the binding of blood platelets to themselves (referred to herein as inhibiting "platelet aggregation"); and/or the binding of compounds or substances, particularly proteins, to the glycoprotein IIb-IIIa complex found in blood platelet membranes (referred to herein as inhibiting "glycoprotein IIb-IIIa binding"). The subject com-

pounds are also useful in retarding or preventing the formation of blood clots or thrombi (referred to herein as inhibiting "thrombosis") and/or the spread of cancer cells throughout the body (referred to herein as inhibiting "cancer metastasis"). The selectivity of these compounds in carrying out the foregoing related activities makes them particularly useful as therapeutic and/or diagnostic agents.

As used throughout this specification, amino acid residues are denoted by the following abbreviations:

Alanine	= Ala
Arginine	= Arg
Asparagine	= Asn
Aspartic acid	= Asp
Cysteine	= Cys
Glutamic acid	= Glu
Glutamine	= Gln
Glycine	= Gly
Histidine	= His
Isoleucine	= Ile
Leucine	= Leu
Lysine	= Lys
Methionine	= Met
Phenylalanine	= Phe
Proline	= Pro
Serine	= Ser
Threonine	= Thr
Tryptophan	= Trp
Tyrosine	= Tyr
Valine	= Val

For maximal activity, the amino acid residues comprising the key tripeptide region, Arg-Tyr-Asp, of the subject invention must be in the L-configuration. The remaining amino acid residues of the subject invention can be present in either the D- or the L-configuration.

As previously noted, in the above Formula I, X¹ and X², and in the above Formula II, X¹, X² and Y, may each independently comprise one or more amino acid residues. The X¹, X² and/or Y amino acid residues, as the case may be, may be linked among themselves, or with each other, in branched, cyclical or straight chain form, although straight chain linkages are preferred. As one skilled in the art would recognize, branched or cyclical chains may be produced by the formation of a peptide bond with amino acid side groups that contain amino or carboxyl moieties. Amino acids containing such side groups include, for example, glutamic acid (carboxyl group), aspartic acid (carboxyl group) and lysine (amide group). Branched or cyclical chains may also be produced through the formation of a covalent disulfide bond between amino acid residues having sulfur-containing side groups, such as cysteine.

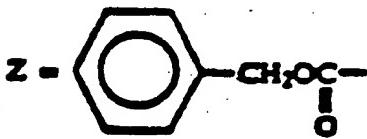
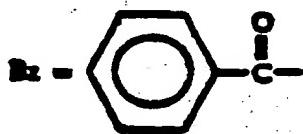
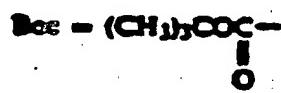
In Formula I, as noted above, m and n may be,

independently, 0 or 1 -- that is, m and n may both be 0 or may both be 1, or m may be 0 when n is 1, or m may be 1 when n is 0. Where both m and n are 0, the subject invention comprises a tripeptide of the sequence Arg-Tyr-Asp, linked, in order, from amino- to carboxy-terminus. Similarly, in Formula II, when each of m, n and o is 0, the subject invention is also directed to the tripeptide Arg-Tyr-Asp.

The R¹ substituent of Formulas I or II may be either an unprotected or protected terminal amino group (amino-terminus). Similarly, R² may be either an unprotected or protected terminal carboxy group (carboxy-terminus).

As used herein, "protected" terminal amino group, refers to a terminal amino group coupled with any of various amino-terminal protecting groups traditionally employed in peptide synthesis. Examples of suitable groups include acyl protecting groups, for example, formyl, acetyl, benzoyl, trifluoroacetyl, succinyl and methoxysuccinyl; aromatic urethane protecting groups, for example, benzyloxycarbonyl; and aliphatic urethane protecting groups, for example, tert-butoxycarbonyl or adamantlyloxycarbonyl. Gross and Mienhofer, eds., The Peptides, Vol. 3, pp. 3-88 (Academic Press, New York, 1981), disclose numerous suitable terminal amino protecting groups.

The following represent preferred amino terminal protecting groups:



The amino acid residues of the X¹, X² and Y substituents having side-chain amino groups, for

example, Lys or Arg, can optionally comprise suitable amino-terminal protecting groups attached to the side chains.

As used herein, "protected" terminal carboxyl group, refers to a terminal carboxyl group coupled with any of various carboxy-terminal protecting groups. As will be readily apparent to one skilled in the art, suitable groups include tert-butyl, benzyl or other acceptable groups linked to the terminal carboxyl group through an ester or ether bond. Amino acid residues of the X¹, X² and Y substituents having acidic or hydroxy side chains can be similarly protected.

The present invention also contemplates physiologically acceptable salts of Formulas I and II. These salts include acid addition salts, for example, salts of hydrochloric acid, hydrobromic acid, acetic acid, trifluoroacetic acid, citric acid, succinic acid, benzene sulfonic acid or other suitable acid addition salts.

Preferable classes of compounds within the scope of Formula I include:

o Compounds wherein

m is 0; and

n is 0.

o Compounds wherein X¹ and X² are in straight chain forms.

o Compounds wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 40.

o Compounds wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 25.

o Compounds wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 10.

o Compounds wherein

X¹ is Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Try;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr;

m is 1; and

n is 1.

o Compounds wherein

X¹ is Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr;

m is 1; and

n is 1.

o Compounds wherein

X¹ is Gln-Val-Gln-Leu-Lys-Gln-Ser-Gly-Pro Gly-Leu-Val-Gln-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Ser-Leu-Thr-Ser-Tyr-Gly-Val-His-Trp-Val-Arg-Leu-Ser-Pro-Gly-Lys-Gly-Leu-Glu-Trp-Leu-Gly-Val-Ile-Trp-Ser-Gly-Gly-Ser-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-Ile-Ser-Arg-Leu-Ser-Ile-Ser-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Lys-Met-Asn-Ser-Leu-Gln-Ala-Asn-Asp-Thr-Gly-Ile-Tyr-Try-Cys-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp Tyr-Trp-Gly-Gln-Gly-Thr-Ser-Val-Thr-Val-Ser-Ser;

m is 1; and

n is 1.

o Compounds wherein

X¹ is Gln-Val-Gln-Leu-Lys-Gln-Ser-Gly-Pro Gly-Leu-Val-Gln-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Ser-Leu-Thr-Ser-Tyr-Gly-Val-His-Trp-Val-Arg-Leu-Ser-Pro-Gly-Lys-Gly-Leu-Glu-Trp-Leu-Gly-Val-Ile-Trp-Ser-Gly-Gly-Ser-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-Ile-Ser-Arg-Leu-Ser-Ile-Ser-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Lys-Met-Asn-Ser-Leu-Gln-Ala-Asn-Asp-Thr-Gly-Ile-Tyr-Tyr-Cys-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp Tyr-Trp-Gly-Gln-Gly-Thr-Ser-Val-Thr-Val-Ser-Ser;

m is 1; and

n is 1.

o Compounds wherein

R¹ is an unprotected terminal amino group; and R² is an unprotected terminal carboxy group.

Preferable classes of compounds within the scope of Formula II include:

o Compounds wherein

m is 0;

n is 0; and

o is 0. o Compounds wherein X¹, and X² and Y are in straight chain forms.

o Compounds wherein the total number of amino acid residues in X¹, and X² and Y, taken together, is less than about 250.

o Compounds wherein the total number of amino acid residues in X¹, and X² and Y, taken together, is less than about 200.

o Compounds wherein the total number of amino acid residues in X¹, and X² and Y, taken together, is less than about 100.

o Compounds wherein the total number of amino acid residues in X¹, X² and Y, taken together, is less than about 50.

o Compounds wherein the total number of amino acid residues in X¹, X² and Y, taken together, is less than about 40.

o Compounds wherein the total number of amino acid residues in X¹, X² and Y, taken together, is less than about 25.

o Compounds wherein the total number of amino acid residues in X¹, X² and Y, taken together, is less than about 10.

o Compounds wherein

X¹ is Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr;

m is 1;

n is 1; and

o is 0.

o Compounds wherein

X¹ is Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr;

m is 1;

n is 1; and

o is 0.

o Compounds wherein

X¹ is Gln-Val-Gln-Leu-Lys-Gln-Ser-Gly-Pro Gly-Leu-Val-Gln-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Ser-Leu-Thr-Ser-Tyr-Gly-Val-His-Trp-Val-Arg-Leu-Ser-Pro-Gly-Lys-Gly-Leu-Glu-Trp-Leu-Gly-Val-Ile-Trp-Ser-Gly-Gly-Ser-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-Ile-Ser-Arg-Leu-Ser-Ile-Ser-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Lys-Met-Asn-Ser-Leu-Gln-Ala-Asn-Asp-Thr-Gly-Ile-Tyr-Try-Cys-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp Tyr-Trp-Gly-Gln-Gly-Thr-Ser-Val-Thr-Val-Ser-Ser;

m is 1;

n is 1; and

o is 0.

o Compounds wherein

X¹ is Gln-Val-Gln-Leu-Lys-Gln-Ser-Gly-Pro Gly-Leu-Val-Gln-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Ser-Leu-Thr-Ser-Tyr-Gly-Val-His-Trp-Val-Arg-Leu-Ser-Pro-Gly-Lys-Gly-Leu-Glu-Trp-Leu-Gly-Val-Ile-Trp-Ser-Gly-Gly-Ser-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-Ile-Ser-Arg-Leu-Ser-Ile-Ser-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Lys-Met-Asn-Ser-Leu-Gln-Ala-Asn-Asp-Thr-Gly-Ile-Tyr-Try-Cys-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;

X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp Tyr-Trp-Gly-Gln-Gly-Thr-Ser-Val-Thr-Val-Ser-Ser;

m is 1;

n is 1; and

o is 0.

o Compounds wherein

R¹ is an unprotected terminal amino group; and R² is an unprotected terminal carboxy group.

Obvious equivalents of the foregoing compounds include compounds comprising less common or modified amino acids, for example, hydroxyproline, hydroxylsine, cystine, thyroxine, norleucine, pyroglutamic acid or other derivatives capable of incorporation into the peptides of the present invention.

The branched, cyclical and straight chain peptides of Formulas I and II can be synthesized using any one of a number of conventional preparative and recovery methods, as will be readily apparent to one skilled in the art.

A preferable synthesis route for the straight chain, especially the smaller, peptides of the invention is the solid phase method. This method is well known in the art and is described, for example, in U.S. Patent Nos. 4,683,291, 4,244,946, 4,305,872, and 4,316,891, and in Merrifield, J. Am. Chem. Soc., 85 2149 (1964), Vale et al, Science 213, 1394-1397 (1981) and Marke et al, J. Am. Chem. Sci., 103, 3178 (1981), the disclosures of which are incorporated herein by reference. Other preparative methods which may be employed include the processes of Houghten, Proc. Natl. Acad. Sci., 82,

5132 (1985), the disclosures of which are also incorporated herein by reference. A preferable synthesis procedure particularly for the smaller branched or cyclical chain peptides, as a skilled artisan would recognize, would include conventional liquid phase processes. The liquid phase processes, as well as other synthesis methods, are described in Principles of Peptide Synthesis, M. Bodansky, ed. (Springer-Verlag, 1984), the disclosures of which are incorporated herein by reference. As would be apparent to one cognizant of the art, for larger peptides, a preferable procedure may include conventional recombinant DNA techniques. Such techniques are discussed in greater detail below. Recombinant techniques as well known and are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor (1982), the disclosures of which are incorporated herein by reference.

Suitable recovery methods are described in the foregoing publications. Other recovery methods which may be employed include those described in Rivier et al., Peptides: The Structure and Biological Function, pp. 125-128 (1979), the disclosures of which are incorporated herein by reference.

The invention further provides compositions and methods for utilizing the subject compounds and compositions in inhibiting fibrinogen binding, platelet aggregation, and glycoprotein IIb-IIIa binding. The ability of the subject compounds to inhibit the foregoing activities makes them useful in inhibiting the physiological process of thrombosis. In addition, in light of their demonstrated activities, the compounds of the present invention may be employed in inhibiting cancer metastasis, an aberrant physiological phenomenon that is believed to require the adhesion of blood platelets to the cancer cells. The specificity of the subject compounds in carrying out these related functions makes them particularly useful as therapeutic and/or diagnostic agents.

In particular, the compositions of the present invention comprise an effective amount of a compound of Formula I and a physiologically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic, diagnostic or other uses are well-known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Gennaro, A.R., ed. (Mack Publishing Co., Easton PA, 1985).

In practicing the methods of the invention, the compounds or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. They can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing in vitro, the compounds or compositions are applied to a cellular solution, that is, a solution containing

the cellular component or components sought to be inhibited. In employing in vivo, the compounds or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is, the dosage levels necessary to achieve the desired result, will be well within the ambit of one skilled in the art. Typically, applications are commenced at lower dosage levels, with dosage being increased until the desired effect is achieved.

Antibodies, both monoclonal and polyclonal, directed to peptide compounds of the present invention are useful in isolation and identification of the subject proteins, and the present invention also pertains to such antibodies. To prepare the subject antibodies, any one of a number of techniques which known in the art can be employed. In one such technique, polyclonal antibodies may be synthesized by injecting an animal (for example, a rabbit) with one or more compounds of the invention. After injection, the animal naturally produces antibodies to these compounds. When the antibody level rises to a sufficient level, antibody-containing blood, called antiserum, is then drawn from the animal, and the compound-specific antibody is isolated from other antibodies in the antiserum by any one of a number of separation techniques (for example, affinity chromatography). Monoclonal antibodies may be prepared using the technique of Kohler and Milstein, Nature 256, pp. 495-497 (1975).

The invention further relates to nucleic acid, preferably DNA, sequences which encode the peptide compounds of the present invention. Such fragments may be synthesized using standard nucleic acid synthesis techniques or, alternatively, may be obtained from genomic material of various sources using conventional genetic engineering protocol. One source of genomic material is the PAC1 hybridoma cell described in Shattil et al., J. Biol. Chem., 260, 11107-11114 (1985), particularly the PAC1- μ heavy chain variable region of that genome. Using standard recombinant DNA techniques, the nucleic acid sequences can be, if desired, incorporated into an appropriate cloning vector, transduced into an appropriate host cell and the proteins encoded by the nucleic acid fragment expressed and collected for further use, thereby providing a potentially convenient method for the

production of the proteins, particularly the lengthy straight chain proteins, of the subject invention. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor (1982), provides a detailed discussion of various recombinant DNA methodologies.

Cloning vector as used herein is defined as a DNA (or RNA) sequence, which allows the incorporation of specific experimental foreign DNA (or RNA), with the combined DNA (or RNA) being introduced into a host cell that can exist in a stable manner and express the protein dictated by the experimental DNA (or RNA). The foreign DNA (or RNA) combined with the vector DNA (or RNA) constitutes a recombinant DNA (or RNA) molecule which is derived from recombinant technology. Cloning vectors may include plasmids, bacteriophage, viruses and cosmids. Expression vectors are defined herein as DNA (or RNA) sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express either prokaryotic or eucaryotic genes in a variety of cells such as bacteria, yeast, insect and mammalian cells. The proteins may also be expressed in a number of virus systems. An appropriately constructed expression vector should contain an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. A promotor is defined as a DNA (or RNA) sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

Finally, the present invention includes a peptide comprising an amino acid sequence corresponding to the amino acid sequence of the variable region of the μ heavy chain of monoclonal antibody PAC1, as shown in Figure 2, and any antibodies incorporating such sequence. The present invention further includes a peptide comprising an amino acid sequence corresponding to the amino acid sequence of the variable region of the μ heavy chain of monoclonal antibody PAC1, as shown in Figure 2, and an amino acid sequence corresponding to the amino acid sequence of the variable region of the χ light chain of monoclonal antibody PAC1, as shown in Figure 1, and any antibodies incorporating such sequences. As will be apparent to those skilled in the art, and as described in the foregoing discussion of peptide synthesis using chemical and recombinant methodologies such as described in Riechmann et al., Nature, Vol. 332, pp. 323-327 (1988), various tech-

niques are available to prepare such peptides. The recombinant DNA techniques can also be applied in the area of antibody manipulation. For a discussion of antibody engineering, see Riechmann et al., Nature, 332, 323-327 (1988), the disclosures of which are herein incorporated by reference.

Most preferably, the invention is directed to monoclonal antibody PAC1. The hybridoma cell line producing this monoclonal antibody is described in Shattil et al., J. Biol. Chem., 260, 11107-11114 (1985), the disclosures of which are hereby incorporated by reference. Both the hybridoma cell PAC1 and the PAC1 monoclonal antibody produced therefrom are discussed in the Examples below. As with the peptides of Formulas I and II, the peptides and monoclonal antibodies of this paragraph are useful as inhibitors of fibrinogen binding, platelet aggregation, and/or glycoprotein IIb-IIIa binding, and, similarly, are effective in the treatment of certain physiological conditions, such as thrombosis and/or cancer metastasis, and the present invention if further directed to these uses.

The invention is further described in the following Examples. These Examples are not to be construed as limiting the scope of the appended Claims.

Examples

Example 1

Cell Culture and Isolation of mRNA

PAC1 hybridoma cells, prepared as described in Shattil et al., J. Biol. Chem., 260, 11107-11114 (1985), were grown in a standard culture medium containing fetal calf serum, and 5×10^8 cells were harvested and resuspended in 4M guanidine thiocyanate as described in Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). Total RNA was isolated after centrifugation through a CsCl cushion and poly A+ RNA was isolated using oligo(dT) cellulose following the procedures set forth in Aviv et al., Proc. Natl. Acad. Sci., U.S.A., 69, 264-268 (1972).

Example 2

Sequencing of PAC1 Immunoglobulin Variable Regions

Using complementary oligonucleotide primers from the 5' region of the mouse μ and χ constant

region mRNAs, (5'-GCTCTCGCAGGAGACGAG and 5'-GGTGGGAAGATGGATACTAGTT, respectively), the sequences of PAC1- μ (light chain) and χ (heavy chain) mRNAs were determined using the direct dideoxy chain termination method as described by Geliebter in Focus, 9, 1-5-8 (1987). Briefly, 20 ng of end-labeled primers were annealed to 10 μ g of poly A+ mRNA at 50°C and primers were extended in the presence of dideoxynucleotides and reverse transcriptase. The products of this reaction were electrophoresed on 6% denaturing polyacrylamide gels that were then exposed to autoradiography. Once approximately 200 bp of sequence had been determined, complementary primers within the variable region about 150 bp upstream of the constant region were synthesized in order to extend the sequence further 5' within the variable region. The primer used in the PAC1- μ reaction was 5'-CATTAGCTTG-CAGACTGTTC and in the PAC1- χ reaction was 5'-ATCTTGAGTGTGAAATCTGT. A small portion, about 50 nucleotides, of the 3' end of the χ mRNA was determined using the modified Maxam and Gilbert technique of Schlobach et al., J. Exp. Med., 164, 407-427 (1986). Figure 1 shows the nucleotide sequence of the variable region of the χ light chain of a PAC1 hybridoma cell, along with the predicted amino acid sequence. Figure 2 shows the nucleotide sequence of the variable region of the μ heavy chain of a PAC1 hybridoma cell, along with the predicted amino acid sequence. In both Figures 1 and 2, the complementary determining regions (CDRs) are shown in boxes. The amino acids numbered according to convention. Kabat E.A., Wu, T.T., Reid-Miller, M., Perry, H.M., Gottesman, K.S., Sequences of Proteins of Immunologic Interest (US DHHS, PHS, NIH 1987).

Example 3

Peptide Synthesis and Analysis

The peptide NH₂-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr-Arg-Tyr-Asp-Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr-COOH, which corresponds to amino acids 93 through 102 of the PAC1- μ variable region (Figure 2) and which is referred to herein as the Arg-Tyr-Asp-21mer, and various other peptides used herein were synthesized by the solid phase method using an Applied Biosystems Inc. 430A automated synthesizer. Boc-amino acids supplied by the manufacturer were used except for Boc-Asp (Chx). Triple couplings were used for Arg³, Ser⁴, Tyr⁷, Tyr⁸, Arg³, Arg¹ and Ala¹ of the Arg-Tyr-Asp-21mer. The remaining residues were incorporated by a double coupling protocol. The peptides were

cleaved from the solid support by the Sn2/Sn1 HF cleavage procedure described in Tam, J. Amer. Chem. Soc., 105, 6442-6455 (1983) and purified by gel filtration (Sephadex G-50F, 50% HOAc) and reverse phase HPLC (Vydac C18, 15 μ , 5 x 30 cm column, 95% A-B to 75% A-B over 60 minutes at a flow rate of 100 ml/min; A = 0.1% TFA-H₂O, B = 0.1% TFA-CH₃CN). Products were characterized by amino acid analyses after acid hydrolysis, fast atom bombardment mass spectrometry (FABMS), nuclear magnetic resonance spectroscopy, and sequence analysis after Edman degradation. Analyses were consistent with the expected structures, with a product purity of \geq 95%.

Example 4

Binding of PAC1 and Fibrinogen to Platelets

The binding of PAC1 monoclonal antibody and fibrinogen to gel-filtered human blood platelets was examined by fluorescence-activated flow cytometry using the procedures of Shattil et al., Blood, 70, 307-315 (1987). Venous blood from normal donors was drawn into 1/7 vol of NIH formula A acid-citrate-dextrose solution, platelet-rich plasma was obtained, and the platelets were gel-filtered into an isotonic HEPES-containing buffer at pH 7.4, as described in Shattil et al., Blood, 68, 1224-1231 (1986). To examine the effect of synthetic peptides on PAC1 binding to activated platelets, 5 x 10⁷ platelets were incubated for 15 min at room temperature in a final vol of 50 μ l in the presence of varying concentrations of (0-100 μ M) of peptide, 40 μ g/ml fluorescein-labeled PAC1 (FITC-PAC1), 10 μ M ADP and 10 μ M epinephrine. Then, 500 μ l of the isotonic buffer was added and antibody binding was quantitated using a flow cytometer, as described in Shattil et al., Blood, 70, 307-315 (1987). Fibrinogen binding to platelets was measured using the same incubation system, except that 50 μ g/ml of purified human fibrinogen as described in Bennett et al., J. Clin. Invest., 64, 1393-1400 (1979) was added and the monoclonal antibody, FITC-9F9, was used instead of FITC-PAC1. Antibody 9F9 is specific for fibrinogen and has been used successfully to measure the amount of fibrinogen bound to platelets. Abrams et al., Blood, 70, 335a (1987). Previous studies have established that the amount of FITC-labeled antibody bound to platelets determined by flow cytometry is related in a linear fashion to the amount of antibody bound determined in a conventional radioligand binding assay. Shattil et al., Blood, 70, 307-315 (1987). The amount of platelet-bound FITC-PAC1 or FITC-9F9 was determined by analyzing 10,000 platelets for

the extent of fluorescence at 488 nm, and binding expressed as the mean fluorescence intensity in arbitrary fluorescence units.

Under these conditions, the Arg-Gly-Asp-21mer peptide inhibited the binding of FITC-PAC1 to platelets with an IC₅₀ of 20-30 μM (as shown in Figure 3A, filled circles). This was similar to the IC₅₀ of the tetrapeptide, NH₂-Arg-Gly-Asp-Ser-COOH (Arg-Gly-Asp-Ser) (Figure 3A, unfilled circle). In five separate experiments, Dixon plot analysis carried out in accordance with Segel, Enzyme Kinetics 465-504 Wiley-Interscience, N.Y. (1975), revealed that the apparent Ki for inhibition of PAC1 binding by Arg-Tyr-Asp-21mer was 10.2 ± 4.3 μM (SE). As a control, an unrelated peptide or similar length having the sequence HN₂-Tyr-Val-Asp-Gly-Asp-Gln-Cys-(Acm)-Glu-Ser-Asn-Pro-Cys-Leu-Asn-Gly-Gly-Met(O)-Cys-(Acm)-Lys-Asp-Asp-Ile-Asn-Ser-Tyr-Gly-Cys-Phe-COOH (unrelated-28mer) (control) had no effect on FITC-PAC1 binding (Figure 3A, unfilled squares). Identical results were obtained when platelets were activated with phorbol myristate acetate instead of ADP and epinephrine (data not shown).

The ability of Arg-Tyr-Asp-21mer to inhibit fibrinogen binding to activated platelets was also tested. Fibrinogen binding was assessed by flow cytometry using a FITC-labeled anti-fibrinogen monoclonal antibody, 9F9. When platelets were activated with ADP plus epinephrine in the presence of 50 μg/ml of fibrinogen, Arg-Tyr-Asp-21mer inhibited fibrinogen binding with an IC₅₀ of 2 μM (Figure 3B, filled circle). The control peptide (unrelated 28 mer) had no effect on 9F9 binding (Figure 3B, unfilled square). In three experiments, Dixon plot analysis showed that this peptide inhibited fibrinogen binding with an apparent Ki of 5.5 ± 3.5 μM.

To examine the specificity of the Arg-Tyr-Asp-21mer peptide in inhibiting PAC1 monoclonal antibody binding, the effect of this peptide on the binding of monoclonal antibodies to other platelet glycoproteins were examined. The Arg-Tyr-Asp-21mer peptide had no effect on the binding of FITC-AP1 to platelet glycoprotein Ib on resting platelets (Figure 4, unfilled circles). In addition, the peptide had no effect on the binding of either FITC-B1B5 to glycoprotein IIb or of FITC-S12 to GMP-140 on activated platelets (Figure 4, filled squares and unfilled triangles, respectively). The peptide had a marked effect on PAC1 binding (Figure 4, filled circles). Antibody S12, described in McEver et al., J. Biol. Chem., 259: 9799 (1984) specific for an α-granule membrane protein (GMP) expressed on the surface of activated platelets. Antibody B1B5 is specific for glycoprotein IIb, and is discussed in Shattil, J. Biol. Chem., 260, 11107-11114 (1985) and Bennett et al., Proc. Natl. Acad. Sci.,

U.S.A., 80, 2417-2421 (1983).

Example 5

Platelet Aggregation Studies

The Effect on Arg-Tyr-Asp-21mer peptide on blood platelet aggregation was examined. The aggregation of gel-filtered platelets was carried out as described in Shattil et al., Blood, 68, 1224-1231 (1986). Platelets (2×10^8 /ml were stirred at 37°C in the presence of human fibrinogen (100 μg/ml) and CaCl₂ (1 mM) and aggregation was initiated by the addition of 10 μM ADP. Aggregation was monitored as a change in light transmittance, and is expressed as the initial rate of aggregation.

In three experiments Arg-Tyr-Asp-21mer inhibited the initial rate of ADP-induced platelet aggregation with an IC₅₀ of approximately 40 μM (Figure 5, filled circles). At 10 μg/ml fibrinogen the IC₅₀ was approximately 10 μM (data not shown).

Example 6

Modifications in the Arg-Tyr-Asp Sequence of the 21mer Peptide

The specific role of the Arg-Tyr-Asp sequence within Arg-Tyr-Asp-21mer was examined by testing three additional peptides identical to the Arg-Tyr-Asp-21mer except for specific modifications in the Arg-Tyr-Asp region. Substitution of the tyrosine in Arg-Tyr-Asp with glycine to produce an Arg-Gly-Asp-21mer increased the inhibitory potency of the peptide by about ten-fold in both the aggregation assay (Arg-Gly-Asp-21mer, Figure 5, filled squares; Arg-Tyr-Asp-21mer, Figure 5, filled circles) and the PAC1 and fibrinogen binding assays (Arg-Tyr-Asp-21mer, Figure 6, filled squares; Arg-Tyr-Asp-21mer, Figure 6, filled squares). On the other hand, substitution of the tyrosine with a D-alanine to produce an Arg-d-Ala-Asp-21mer, or inversion of the Arg and Asp residues to produce an Arg-Tyr-Asp-21mer substantially reduced the inhibitory capacity of the peptide (Arg-d-Ala-Asp-21mer, Figure 6, unfilled circles; Asp-Tyr-Arg-21mer, Figure 6, filled triangles). The Arg-Gly-Asp-Ser-tetramer is shown in Figure 6 as the unfilled square.

These specific modifications of the Arg-Tyr-Asp sequence within the Arg-Tyr-Asp-21mer peptide clearly establish that this sequence of three amino acids is of significance to the functional properties of the entire peptide.

Various modifications of the invention in addi-

tion to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended Claims.

Claims

1. A compound of the formula
 $R^1-(X^1)_n\text{-Arg-Tyr-Asp-(X}^2\text{)}_m\text{-R}^2$
 and physiologically acceptable salts thereof, wherein:
 X¹ and X², independently, are 1 to about 50 amino acid residues, provided that the total number of amino acid residues in X¹ and X², taken together, is equal to or less than about 50;
 m and n, independently, are 0 or 1;
 R¹ is a protected or unprotected terminal amino group; and
 R² is a protected or unprotected terminal carboxy group.
 2. A compound according to Claim 1 wherein
 m is 0; and
 n is 0.
 3. A compound according to either of Claims 1 or 2 wherein X¹ and X² are in straight chain form.
 4. A compound according to any preceding Claim wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 40.
 5. A compound according to any of Claims 1 to 3 wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 25.
 6. A compound according to any of Claims 1 to 3 wherein the total number of amino acid residues in X¹ and X², taken together, is less than about 10.
 7. A compound according to Claim 1 wherein
 X¹ is Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;
 X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr;
 m is 1; and
 n is 1.
 8. A compound according to Claim 1 wherein
 X¹ is Gln-Val-Gln-Leu-Lys-Gln-Ser-Gly-Pro-Gly-Leu-Val-Gln-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Ser-Leu-Thr-Ser-Tyr-Gly-Val-His-Trp-Val-Arg-Leu-Ser-Pro-Gly-Lys-Gly-Leu-Glu-Trp-Leu-Gly-Val-Ile-Trp-Ser-Gly-Gly-Ser-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-Ile-Ser-Arg-Leu-Ser-Ile-Ser-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Lys-Met-Asn-Ser-Leu-Gln-Ala-Asn-Asp-Thr-Gly-Ile-Tyr-Tyr-Cys-Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr;
 X² is Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr-Trp-Gly-Gln-Gly-Thr-Ser-Val-Thr-Val-Ser-Ser;
 m is 1; and
 n is 1.
 9. A compound of the formula
 $R^1-(X^1)_n\text{-Arg-Tyr-Asp-(Y}_o(x^2)\text{)}_m\text{-R}^2$
- and physiologically acceptable salts thereof, wherein:
 X¹ and X², independently, are 1 or more amino acid residues;
 Y is 1 or more amino acid residues, provided that Y does not include a Ser or Ala residue;
 m, n and o, independently, are 0 or 1, provided that when m is 1, o must be 1
 R¹ is a protected or unprotected terminal amino group; and
 R² is a protected or unprotected terminal carboxy group; and wherein said compound inhibits fibrinogen binding, platelet aggregation, thrombosis, cancer metastasis and/or glycoprotein IIb-IIIa binding.
10. A composition for inhibiting, fibrinogen binding, platelet aggregation, thrombosis, and/or cancer metastasis comprising an effective amount of a compound of any preceding Claim and a physiologically acceptable carrier or diluent therefor.
 11. A composition for inhibiting binding of a substance to a blood platelet membrane glycoprotein IIb-IIIa complex comprising an effective amount of a compound of any of Claims 1 to 9 and a physiologically acceptable carrier diluent.
 12. A method of inhibiting fibrinogen binding, platelet aggregation, comprising administering to the platelets an effective amount of a compound of any of Claims 1 to 9.
 13. A method of inhibiting binding of a substance to a blood platelet membrane glycoprotein IIb-IIIa complex comprising administering to the complex an effective amount of a compound of any of Claims 1 to 9.
 14. An antibody to a compound of Claim 1.
 15. A nucleic acid fragment comprising a nucleotide sequence which encodes a compound of any of Claims 1 to 9.
 16. A peptide comprising an amino acid sequence corresponding to the amino acid sequence shown in Figure 2.
 17. A peptide according to Claim 16 further comprising an amino acid sequence corresponding to the amino acid sequence as shown in Figure 1.
 18. A monoclonal antibody comprising an amino acid sequence corresponding to the amino acid sequence shown in Figure 2.
 19. A monoclonal antibody according to Claim 18, further comprising an amino acid sequence corresponding to the amino acid sequence shown in Figure 1.
 20. The use in the manufacture of a medicament effective *in vivo* for inhibiting fibrinogen binding, platelet aggregation, thrombosis, and/or cancer metastasis which comprises a composition according to any of Claims 1 to 9

FIGURE 1

PAC1- κ Variable Region

60	CAT GTC TGC ATG ACC CAA ACT CCA CTC TCC CTC CCT GTC AGT CTT CGA GAT CAA CCC CCC	10	Val Leu Met Thr Glu Thr Pro Leu Ser Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Glu Ala Ser
90	ATC TCT TGC AGA TCT AGT CAG ACC ATT GTA CAT AGT ATT CGA AAC ACC TAT TTA GAA TGG	120	
27	A B C D E	28	CDR1 CDR2
Ile Ser Cys Arg Ser Ser Glu Ser Ile Val His Ser Ile Ser Lys Ser Gly Asn Thr Tyr Leu Glu Trp	35		
150	TAC CTG CAG AAA CCA GCC CAG TCT CCA AUG CTC CTC ATC TAC TAC AAA GTC TCC AAC CCA TTT	180	
45	Tyr Leu Glu Lys Pro Gly Glu Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe	210	
65	TCT GGG GTC CCA GAC AGG TTC ACT GCC AGT GCA TCA GGT ACA CTC AUG ATC	240	
85	Ser Glu Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	270	V 300
107	ACC AGA GTC GAG CCT GAG GAT CTC CGA GTC TAT TAC TCC TCG CAA CGT TCA CAT GTC CCC	336	
107	Ser Arg Val Glu Ala Asp Leu Gly Val Tyr Tyr Cys Tri Glu Gly Ser His Val Pro	95	CDR3
	Tyr		

FIGURE 2

PAC1- μ Variable Region

CAG	GTC	CAC	CTG	AGC	TCA	GCA	CCT	GCG	CTA	GTC	CAG	CCC	TCA	CAG	AGC	CTG	TCC	TCC	ATC	60
Gln	Val	Gln	Leu	Lys	Gln	Ser	Gly	Pro	Gly	Leu	Val	Gln	Pro	Ser	Gln	Ser	Leu	Ser	Ile	20
ACC	TCC	ACA	GTC	TCT	GGT	TTC	TCA	TTA	ACT	ACC	TAT	GCT	GTA	CAC	TGC	GTT	CCC	GTC	TCT	120
Thr	Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Thr	Ser	Tyr	Gly	Val	His	Trp	Val	Arg	Leu	Ser	40
CCA	CCA	AGC	GCT	CTG	GAG	TGG	CTG	CGA	GTC	ATA	TGG	AGT	GGT	GGA	AGC	ACA	GAC	TAT	TAT	180
Pro	Gly	Lys	Gly	Leu	Glu	Trp	Leu	Gly											CDR2	60
GCA	GCT	TTC	ATA	TCC	AGA	CTG	AGC	ATC	AGC	ATA	TCC	AGC	AGC	CAA	GTT	TTC	TTC	TTC	240	
Ala	Ala	Phe	Ile	Ser	Arg	Leu	Ser	Ile	Ser	Lys	Asp	Ala	Ser	Lys	Ser	Gln	Val	Phe	Phe	80
AAA	ATG	AAC	AGT	CTG	CAA	GCT	ATT	GAC	ACA	GCG	ATA	TAT	TAC	TGT	TCC	AGA	AGA	AGC	CCC	300
82	A	B	C	83	83	87													97	
Lys	Met	Leu	Ser	Gln	Ala	Ala	Ala	Asp	Thr	Gly	Ile	Tyr	Tyr	Tyr	Cys	Ala	Arg	Arg	Ser	Pro
CDR3	100	A	B	C	D	E	F	G	H	I	J	K	101						106	
Ser	Tyr	Tyr	Arg	Tyr	Asp	Gly	Ala	Gly	Pro	Tyr	Tyr	Ala	Met	Arg	Tyr	Trp	Gly	Gln	Gly	360
ACC	TCA	GTC	ACC	GTC	TCA	GCG	GCG	GGT	CCT	TAT	GCT	ATG	GAC	TAC	TGG	GCT	CM	CCA	378	
Thr	Ser	Val	Thr	Val	Ser	S	r												113	

V D
330D34

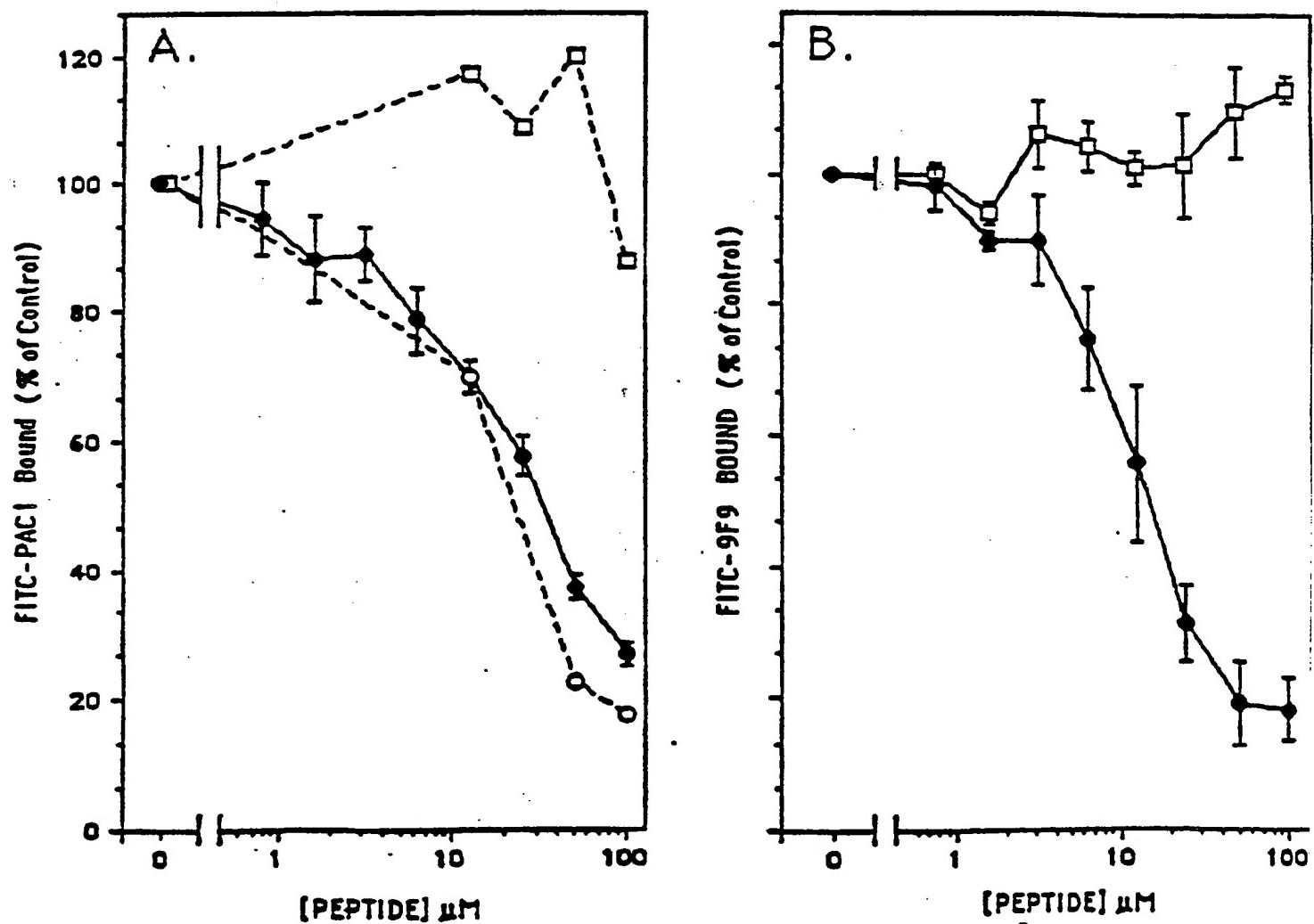
FIGURE 3**Effect of Arg-Tyr-Asp-21mer on Binding of
PAc1 and Fibrinogen to Activated Platelets**

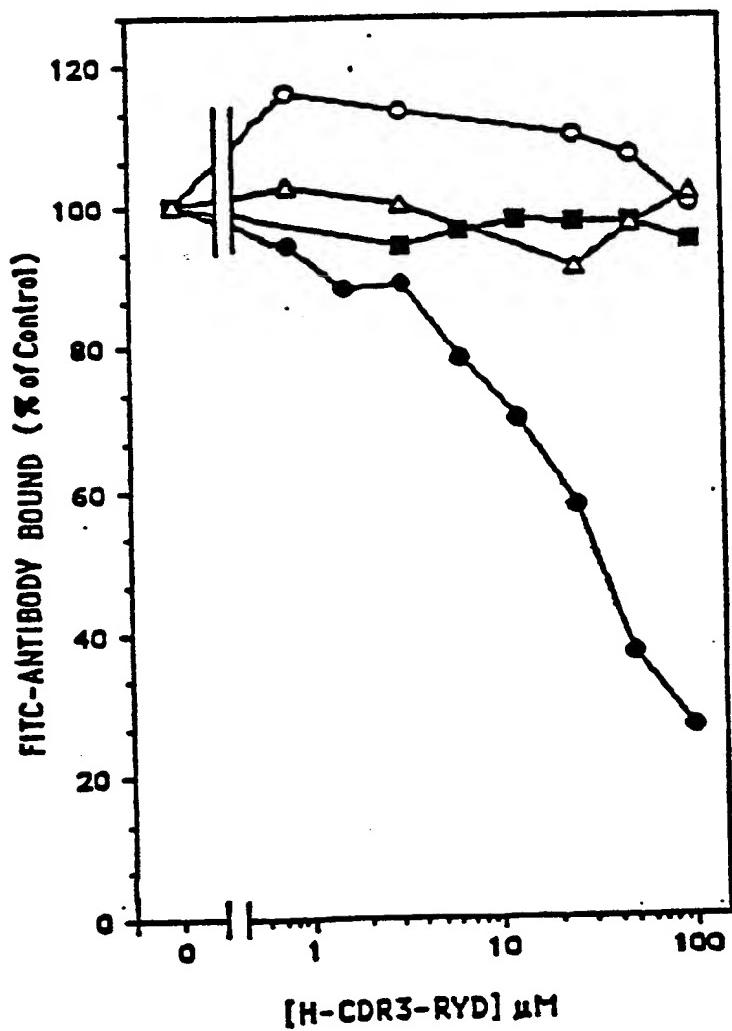
FIGURE 4Eff ct of Arg-Tyr-Asp-21mer n Binding f
Monoclonal Antibodies to Platelets

FIGURE 5

**Effect of Arg-Tyr-Asp-21mer on
ADP-Induced Platelet Aggregation**

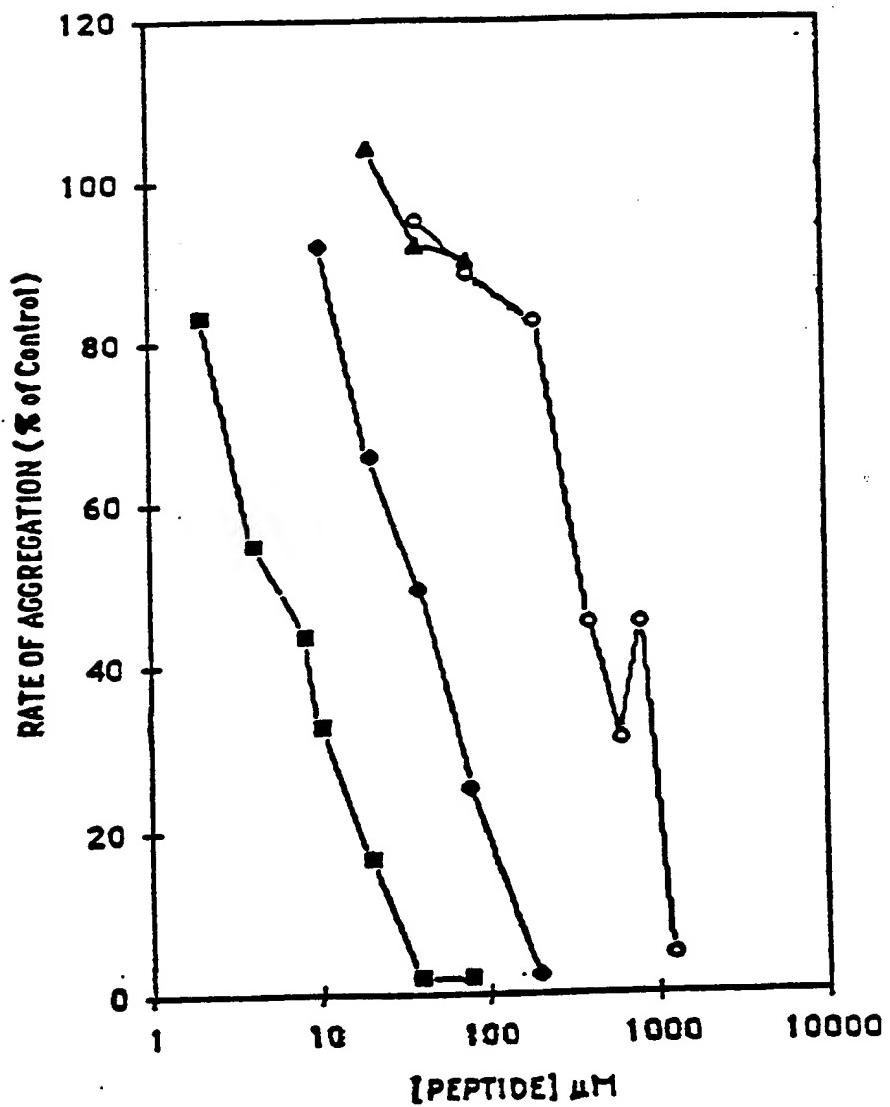
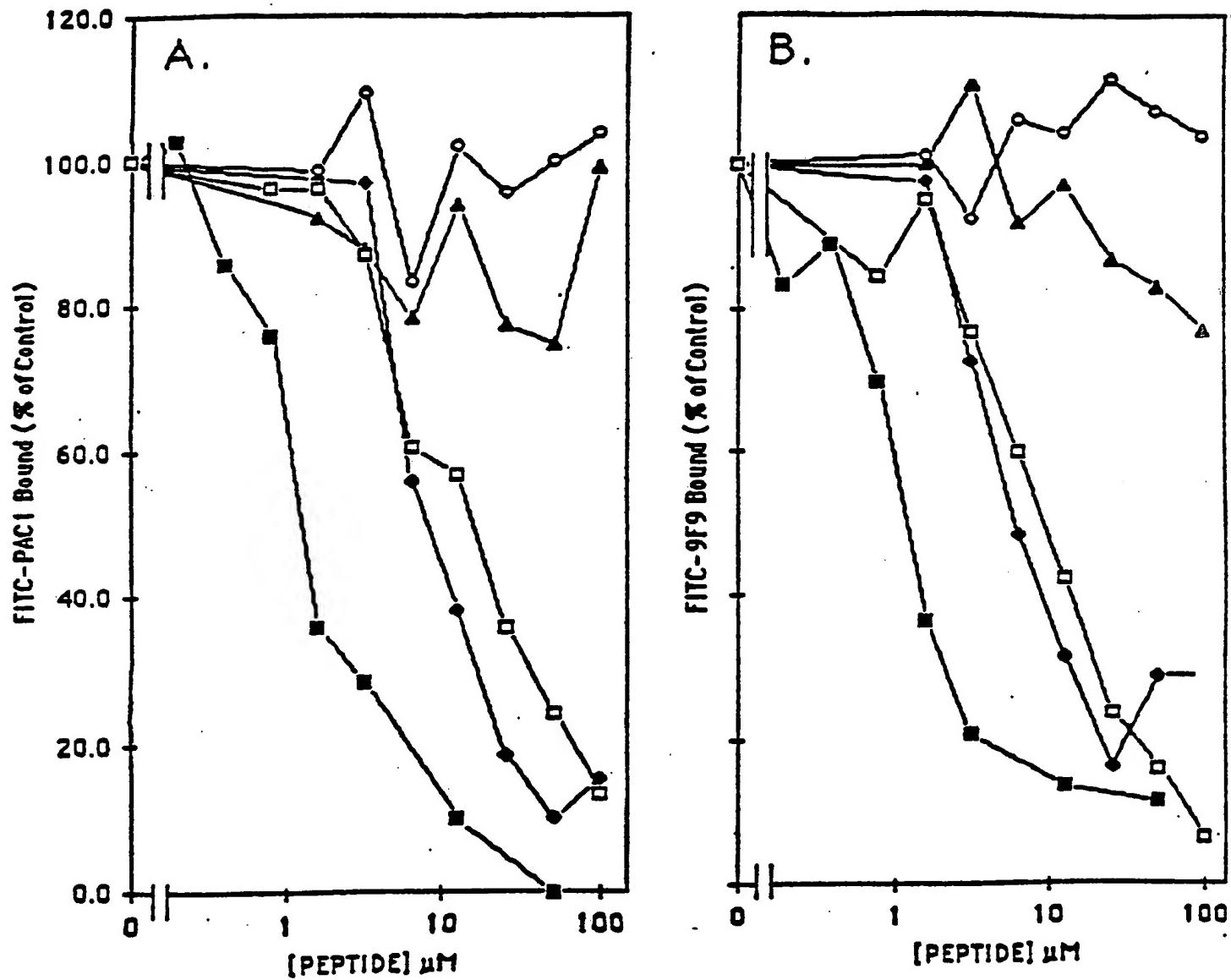


FIGURE 6

Effect of Arg-Tyr-Asp-21mer on the Binding of PAC1 and 9F9 to Activated Platelets



EP 0 368 486 A3



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 368 486 A3

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 89310620.3

(22) Date of filing: 17.10.89

(51) Int. Cl.⁵: **C07K 5/08, C07K 7/06,
C07K 7/10, C12P 21/00,
C12N 15/00, A61K 37/02,
C07K 13/00**

(33) Priority: 10.11.88 US 269583

(43) Date of publication of application:
16.05.90 Bulletin 90/20

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(88) Date of deferred publication of the search report:
30.01.91 Bulletin 91/05

(71) Applicant: **MERCK & CO. INC.NC.
126, East Lincoln Avenue P.O. Box 2000
Rahway New Jersey 07065-0900(US)**

Applicant: **THE TRUSTEES OF THE
UNIVERSITY OF PENNSYLVANIA
c/o Office of Research Administration 133
South 36th Street Suite 300
Philadelphia Pennsylvania 19104-3246(US)**

(72) Inventor: **Shattil, Sanford J.
510 Merwyn Road
Narberth Pennsylvania 19072(US)**
Inventor: **Taub, Rebecca A.
403 Great Spring Road
Rosemont Pennsylvania 19010(US)**
Inventor: **Friedman, Paul A.
403 Great Spring Road
Rosemont Pennsylvania 19010(US)**

(74) Representative: **Stebbing, Peter John Hunter
Stebbing & Co. 45 Lancaster Mews
Lancaster Gate, London W2 3QQ(GB)**

(54) **Novel inhibitors of platelet binding.**

(57) Peptides comprising the tripeptide sequence of arginine-tyrosine-aspartic acid, linked from amino- to carboxy-terminus, are inhibitors of fibrinogen binding, platelet aggregation, glycoprotein IIb-IIIa binding and related aberrant and normal physiological conditions.



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT
which under Rule 45 of the European Patent Convention
shall be considered, for the purposes of subsequent
proceedings, as the European search report

Application number
EP 89 31 0620

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	<p>THE JOURNAL OF CELL BIOLOGY, vol. 105, no. 5, November 1987, pages 2403-2408, The Rockefeller University Press; P.F. GOETINCK et al.: "The tandemly repeated sequences of cartilage link protein contain the sites for interaction with hyaluronic acid"</p> <p>* Page 2404, column 1 *</p> <p>--</p>	1,3,4 5,6	C 07 K 5/08 C 07 K 7/06 C 07 K 7/10 C 12 P 21/00 C 12 N 15/00 A 61 K 37/02 C 07 K 13/00
P,X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 1, 5th January 1989, pages 259-265, The American Society for Biochemistry and Molecular Biology, Inc.;</p> <p>R. TAUB et al.: "A monoclonal antibody against the platelet fibrinogen receptor contains a</p>	./.	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.</p> <p>Claims searched completely: 1-11,14-20</p> <p>Claims searched incompletely: 12,13</p> <p>Claims not searched: 12,13</p> <p>Reason for the limitation of the search:</p> <p>Method for treatment of the human or animal body by surgery or therapy (See art. 52(4) of the European Patent Convention)</p>			
Place of search THE HAGUE		Date of completion of the search 07-11-1990	Examiner NOVOA Y SANJURJO
CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application number

EP 89 31 0620
-2-

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	<p>sequence that mimics a receptor recognition domain in fibrinogen"</p> <p>* Whole article *</p> <p>--</p>	1-20	
D,A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 26, 15th September 1988, pages 12948-12953, The American Society for Biochemistry and Molecular Biology, Inc.;</p> <p>J.S. BENNETT et al.: "Interaction of fibrinogen with its platelet receptor"</p> <p>--</p>		TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
D,A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, 15th September 1985, pages 11107-11114, The American Society of Biological Chemists, Inc.;</p> <p>S.J. SHATTIL et al.: "Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation"</p> <p>-----</p>		